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MPP^+ toxicity and plasma membrane dopamine transporter: study using cell lines expressing the wild-type and mutant rat dopamine transporters

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Abstract

The Parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) causes specific cell death in dopaminergic neurons after accumulation by the dopamine transporter (DAT). COS cells, a non-neuronal cell line insensitive to high doses of MPP^+, becomes sensitive to MPP^+ when transfected with the rat DAT cDNA. We analyzed the bi-directional transport of MPP^+ and its toxicity in several cell lines expressing wild or mutant DATs. Cell death in COS cells expressing wild DAT by exposure to MPP^+ was concentration-dependent and cocaine-reversible. Increased wild DAT expression caused higher sensitivities to the toxin in HeLa cells. Although several mutant DATs demonstrated greater transport activity than the wild-type, they displayed similar or lower sensitivity to MPP^+ toxicity. Reverse transport of preloaded [^{3}H]MPP^+ through DAT was facilitated in COS cells expressing certain mutant DATs, which consistently displayed less sensitivity to MPP^+ toxicity. These results suggest that re-distribution of MPP^+ due to influx/efflux turnover through the transporter is a key factor in MPP^+ toxicity. 0167-4889/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Dopamine; Transporter; MPP^+; Parkinsonism; Neurotoxin; Efflux

1. Introduction

1-Methyl-4-phenylpyridinium (MPP^+), an active metabolite of the Parkinsonism-inducing neurotoxin *N*-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MTPT), causes specific cell death in dopaminergic neurons after accumulation by the dopamine transporter (DAT) ([1–4], see review [5]). Although numerous attempts have been made to clarify the action of MPP^+, several questions remain to be answered, including species differences in sensitivity to MPTP toxicity. One reason rats are relatively insensitive to MPTP may be the low activity of MAO_B, which catalyzes MPTP to MPP^+ [5]. At present, however, this is not an entirely convincing explanation.

Molecular cloning of DAT opens new avenues for investigating the action of MPP^+ [6,7]. We have demonstrated that COS cells, a non-neuronal cell line insensitive to MPP^+ even at high concentrations, become sensitive to MPP^+ when transfected with the rat DAT [8]. Pifl et al. [9] demonstrated MPP^+ toxicity in different cell lines expressing human DAT. Using DAT and norepinephrine transporter (NET),

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they analyzed the properties of the transporters that may be involved in MPP^+ toxicity and suggested the importance of the initial velocity of uptake by the transporter rather than affinity for substrate [10]. However, this speculation have not been confirmed experimentally.

Recently, we reported the reverse transport of dopamine (DA) and MPP^+ through expressed rat DAT in COS cells [11] and in *Xenopus laevis* oocyte [12]. The nature of the efflux of MPP^+ through the DAT expressed in COS cells was quite different from that of DA: persistent efflux of [^{3}H]MPP^+ was observed and more than 70% of preloaded [^{3}H]MPP^+ was released during initial 15 min incubation. These results suggested that re-distribution of accumulated MPP^+ through reverse transport across the plasma membrane may occur quickly, affecting toxicity.

To analyze the relationship between transporter activity and MPP^+ toxicity, we examined the MPP^+ toxicity in COS cells expressing wild and various mutant DATs including 7S-A2 mutant DAT, which revealed a marked increase in initial velocity (Vmax) for MPP^+ uptake [13]. Contrary to our prediction, sensitivity to MPP^+ toxicity decreased in the COS cells expressing the 7S-A2 mutant DAT. To resolve this apparent discrepancy, we focused on the role of MPP^+ release through the DAT in the expression of toxicity in several cell lines expressing wild or mutant DATs with different kinetic properties.

2. Materials and methods

2.1. Expression of DAT

COS cells were transfected with 20 μg pcDNAI or pcDNA3 carrying wild or mutant rat DAT cDNA by electroporation [14]. The cells were then diluted in culture medium (DMEM + 10% FCS), plated in 24-well plate (for uptake and efflux assay) or 96-well plate (for MPP^+ toxicity assay), and cultured for 2–4 days.

Stable cell lines expressing DAT were made by transfection of HeLa cells with pcDNA3 carrying rat DAT and selected by G418 (600 μg/ml) and [^{3}H]DA uptake [15]. One month after G418 treatment, 4 of 25 clones resistant to G418 treatment showed high to low ability to take up [^{3}H]DA. Those were designated HeLa/DAT B5, D2, B2, C5, respectively, and analyzed further.

2.2. MPP^+ toxicity assay

One-day cultures after electroporation were washed with Hank's balanced salt solution (HBSS), then incubated in culture medium containing different concentrations of MPP^+ with or without 100 μM cocaine under culture condition for 2 days. Cells were then subjected for microscopic analysis. The quantitative toxicity assay used WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) (Dojindo, Kumamoto, Japan), a water soluble folmazan dye, which reflects mitochondrial respiratory activity [16]. Optical density (OD) was measured using microplate reader (Tosoh, Tokyo, Japan) at 450 nm, 1, 2 and 3 h after incubation with WST-1 (10 μl of 5 mM WST-1 per 100 μl of culture).

In some experiments, release of lactate dehydrogenase (LDH) into the culture medium was measured for evaluation of MPP^+ toxicity. After a 1-h incubation of cells with various concentrations of MPP^+, cells were washed three times with HBSS, incubated with toxin-free HBSS for 3 h, then the LDH released into the incubation solution was measured colorimetrically according to the manufacturer's protocol (Wako, Tokyo, Japan).

2.3. Uptake and efflux assay

Procedures for assaying uptake and efflux of radiolabeled ligands have been described elsewhere [11]. Briefly, transfected and cultured cells were washed with Krebs Ringer HEPES-buffered solution (KRH) containing 0.1% BSA, incubated with 100 nM [^{3}H]DA (888 GBq/mmol, NEN DuPont) or [^{3}H]MPP^+ (2956.3 GBq/mmol, NEN DuPont) at 37°C for 10 min. The [^{3}H]DA or [^{3}H]MPP^+ uptake was separated by rapid wash of cells with ice-cold KRH, and the radioactivity extracted by 2 N NaOH was determined by liquid scintillation counting. Non-specific uptake was determined in the presence of 100 μM cocaine.

For the efflux study, cells were preloaded with 10 nM [^{3}H]MPP^+ at 37°C for 30 min, washed with

KRH, then incubated in fresh KRH containing test compounds. The incubation medium was separated rapidly from the culture well, and the radioactivity in the medium along with that remaining in the cells were separately measured by liquid scintillation counting. Release of [^{3}H]MPP$^+$ was expressed as a percent of total cell content initially present at the start of incubation (sum of those in the medium and in the cells at the end of incubation).

2.4. Construction of mutant DATs by site-directed mutagenesis

In vitro mutagenesis of rat DATs was performed by the method described previously [13,14], except Y533A and Y533F [15].

Two serines S353 and S355 located in putative 7th transmembrane region were replaced by alanine (designated 7S-A2) [14]. Tyrosine-251 and tyrosine-273 located in the putative 4th and 5th transmembrane regions, respectively, were replaced by alanine, and designated Y251A, Y273A.

Y533A and Y533F, in which tyrosine-533 located in the putative 11th transmembrane region was replaced by alanine (Y533A) or phenylalanine (Y533F), were constructed by PCR-based mutagenesis according to manufacturer's protocol (Stratagene) [15].

3. Results

In the present study, we examined the relationship between MPP$^+$ toxicity and MPP$^+$ transport through the plasma membrane via DAT in several cell lines expressing wild and mutant DATs. The aim of using mutant DATs with different transport properties was to isolate the transporter functions important for MPP$^+$ toxicity.

We have demonstrated that COS cells, a non-neuronal cell line insensitive to high doses of MPP$^+$, become sensitive to MPP$^+$ toxicity when transfected with the rat DAT cDNA [8]. Fig. 1 shows the effect of MPP$^+$ on cell viability in COS cells expressing the wild DAT. Using WST-1, a dye indicator of mitochondrial respiration, quantitative analysis of MPP$^+$

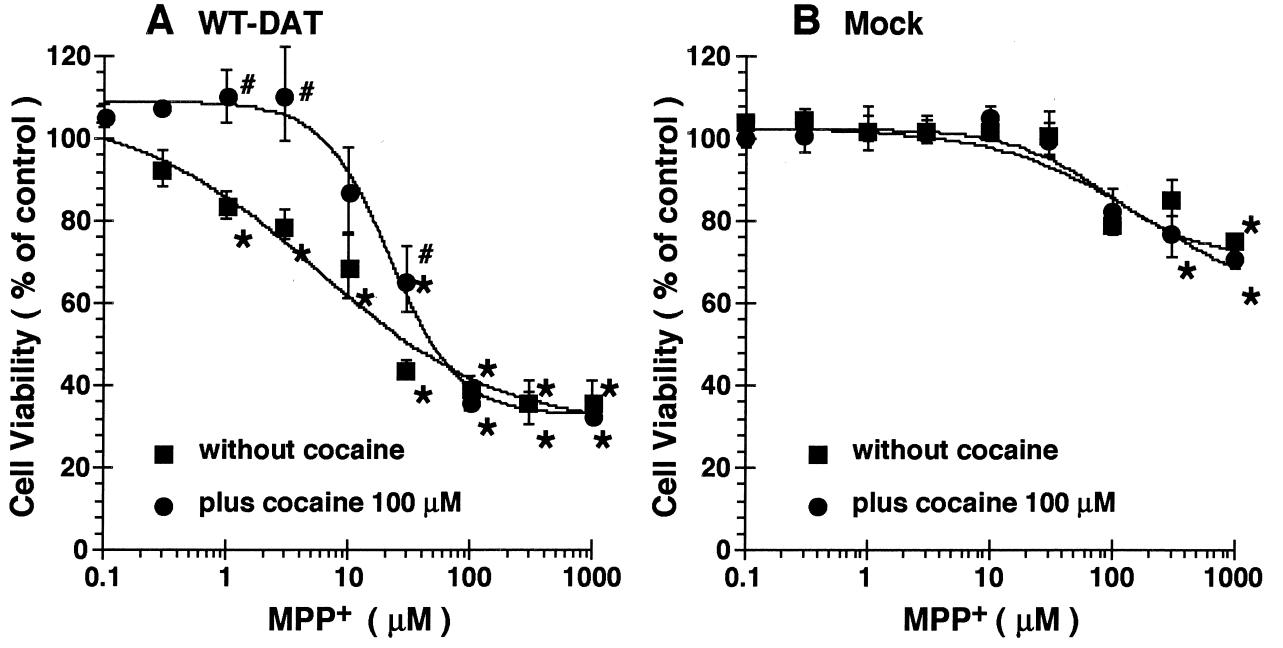

Fig. 1. Effects of MPP$^+$ exposure on cell viability in COS cells expressing wild rat DAT. Quantitative analysis of MPP$^+$ toxicity was performed using WST-1. (A) Two days exposure of COS cells expressing wild rat DAT to various concentrations of MPP$^+$ with or without 100 μM cocaine. (B) MPP$^+$ toxicity in control COS cells mock-transfected with pcDNA3 vector. Points and bars represent the mean ± S.E.M., $n=4$. *Significantly different from control (without MPP$^+$) at $P<0.01$ analyzed by Student's t-test. $^{\#}P<0.05$ vs. without cocaine. Following statistical analyses were performed in the same way.

toxicity was performed in COS cells. When cells were transfected with rat DAT, MPP^+ caused cell death in a concentration-dependent fashion. Cocaine competitively blocked the effect. Mock-transfected COS cells showed less sensitivity to MPP^+ toxicity and cocaine was without effect.

Increased DAT expression in selected clones of the transfected HeLa cells revealed greater sensitivity to

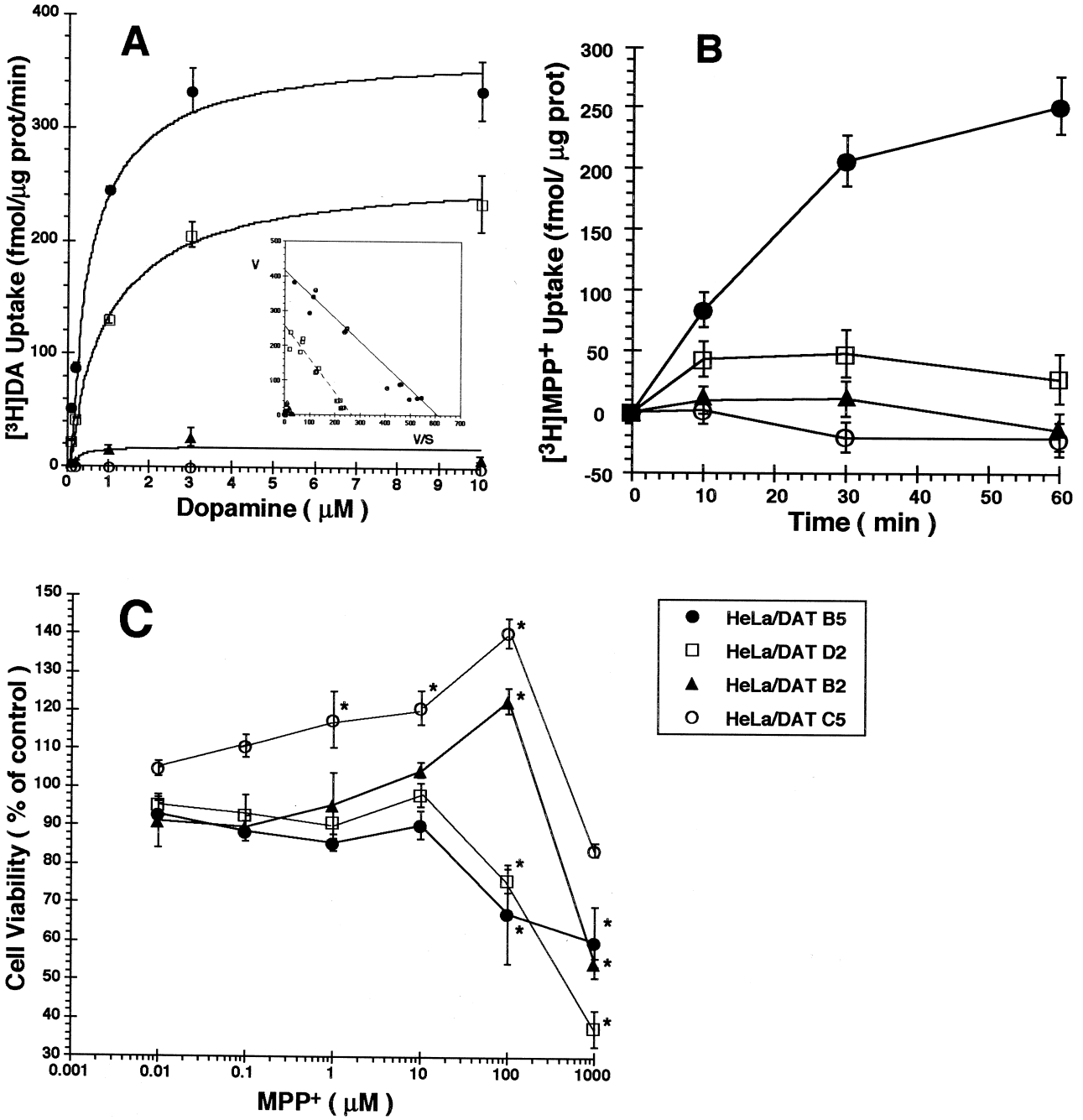

Fig. 2. MPP^+ toxicity in HeLa cells stably expressing wild rat DAT. (A) Kinetics of [^{3}H]dopamine uptake in HeLa cells differently expressing the rat DAT, clone B5, D2, B2 and C5. (B) Time-course of [^{3}H]MPP^+ uptake in four different clones of HeLa cells. (C) MPP^+ toxicity in four different clones of HeLa cells, assessed by WST-1 and expressed as % of control (without MPP^+). Points and bars represent the mean ± S.E.M., $n = 3$–4. *$P < 0.05$ vs. control (without MPP^+).

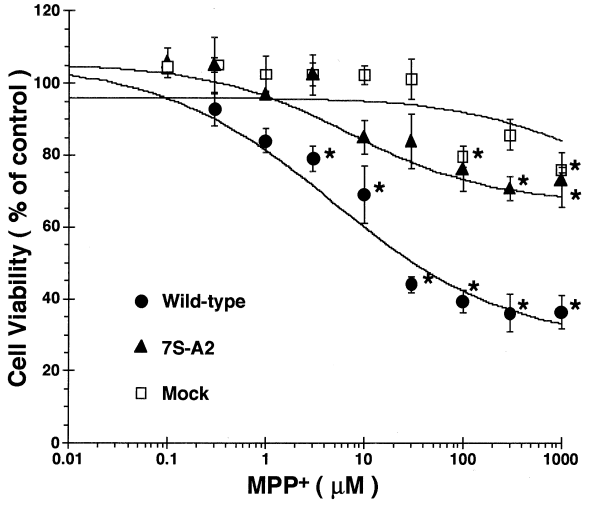

Fig. 3. MPP^+ toxicity in COS cells expressing wild or 7S-A2 mutant DAT. After transfection of COS cells with wild or 7S-A2 mutant DAT, cells were initially cultured for 24 h, then further incubated with 0.1–1000 μM MPP^+ for 2 days. After the MPP^+ treatment, cell number was assessed by WST-1. Points and bars represent the mean ± S.E.M., $n=4$. $^*P<0.05$ vs. control (without MPP^+).

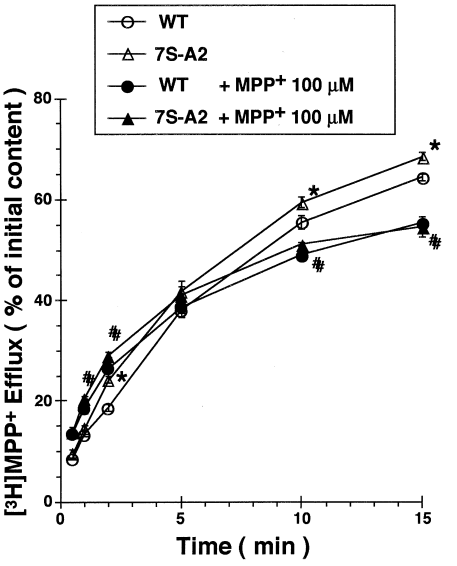

Fig. 4. Efflux of $[^3H]MPP^+$ through the transporter in COS cells expressing wild or 7S-A2 mutant DAT. The DAT-expressed COS cells preloaded with $[^3H]MPP^+$ were washed and incubated in the fresh medium with or without 100 μM unlabeled MPP^+ for various times. After incubation, the medium was separated from cells, and the radioactivity in the medium and that remaining in the cells were measured by liquid scintillation counting. $[^3H]MPP^+$ efflux was expressed as % of initial content prior to incubation. Points and bars represent the mean ± S.E.M., $n=3$. $^*P<0.05$ vs. wild-type (WT), $^{\#}P<0.05$ vs. control (MPP^+ minus) of each WT or 7S-A2 DAT.

MPP^+ toxicity (Fig. 2C) in parallel to DA uptake velocity (Fig. 2A) and MPP^+ transport (Fig. 2B). These results suggest that transporter expression or transport activity is related to MPP^+ toxicity.

Given this rationale, we first examined MPP^+ toxicity in COS cells expressing the 7S-A2 mutant DAT, which shows increased initial velocity (V_{max}) for MPP^+ uptake [13]. Contrary to our hypothesis, these cells showed less sensitivity to MPP^+ toxicity, as shown in Fig. 3. These results indicate that uptake velocity is not unnecessarily related to MPP^+ toxicity. Although MPP^+ must be taken up into cells to be toxic, the kinetics of uptake over a short time cannot predict toxicity.

To resolve this apparent discrepancy, we examined MPP^+ release via reversal of transport by DAT and MPP^+ toxicity in COS cells expressing the various mutant DATs, which isolated the different transport properties.

Table 1
Functional characterization of Y251A, Y273A mutant dopamine transporter expressed in COS cells

Transporter	$[^3H]DA$ uptake		$[^3H]MPP^+$ uptake		$[^3H]CFT$ binding[a]	
	K_m (μM)	V_{max} (pmol/μg protein/min)	K_m (μM)	V_{max} (fmol/μg protein/min)	K_d (nM)	B_{max} (fmol/μg protein)
Wild	2.09 ± 0.15	3.96 ± 0.16	38.0 ± 5.1	353.7 ± 19.7	89.4 ± 56.2	97.4 ± 43.0
Y251A	0.92 ± 0.07[b]	0.74 ± 0.03[b]	9.0 ± 1.1[b]	57.1 ± 2.0[b]	460.7 ± 288.5	73.1 ± 39.9
Y273A	2.03 ± 0.07	0.57 ± 0.05[b]	35.2 ± 4.2	96.4 ± 4.6[b]	272.5 ± 90.1	129.9 ± 35.3

Values represent the mean ± S.E.M ($n=3$).

[a]Binding of $[^3H]CFT$ ((−)-2β-carbomethoxy-3β-(4-fluorophenyl)-tropane), a cocaine analog, was performed using whole cell cultures placed on ice in KRH with 4 nM $[^3H]CFT$ (3085.8 GBq/mmol, NEN DuPont), as previously described [13,14]. Specific binding was calculated by subtracting the non-specific binding determined in the presence of 100 μM cocaine.

[b]Significantly different from Wild at $P<0.05$.

The differences in reverse transport through wild versus 7S-A2 mutant DATs are shown in Fig. 4. 7S-A2 mutant DAT demonstrated a slight increase in efflux of MPP^+ over 15 min incubated periods. When unlabeled MPP^+ was present in the extracellular medium, release of [^{3}H]MPP^+ decreased in COS cells expressing wild DAT. This change could reflect an increase in homo-exchange, assuming that the ratio of intracellular [^{3}H]MPP^+ to unlabeled MPP^+ decreased during incubation periods due to the uptake of unlabeled MPP^+, resulting in the net increase of intracellular MPP^+ (unlabeled MPP^+ plus [^{3}H]MPP^+). In COS cells expressing 7S-A2 mutant DAT, the addition of unlabeled MPP^+ to the incubation medium caused a decrease in [^{3}H]MPP^+ efflux. In light of the above hypothesis, these results suggest that 7S-A2 mutant DAT facilitates homo-exchange. Similar association and dissociation constant for the binding of MPP^+ to wild and 7S-A2 mutant DATs were observed (data not shown). Taken together with MPP^+ toxicity data comparing wild and 7S-A2 mutant DAT-expressing COS cells, efflux of MPP^+ through the transporter may be important factor in its toxicity.

Table 1 shows the uptake of [^{3}H]DA and [^{3}H]MPP^+ and [^{3}H]CFT binding in COS cells expressing the Y251A and Y273A mutant DATs. Y251A mutant DAT showed an increased affinity (K_m) for [^{3}H]DA and [^{3}H]MPP^+ uptake with decreased V_{max}, whereas Y273A mutant DAT showed a decrease in V_{max} for [^{3}H]DA and [^{3}H]MPP^+ uptake without changing K_m. The dissociation constant (K_d) for [^{3}H]CFT binding was increased in COS cells ex-

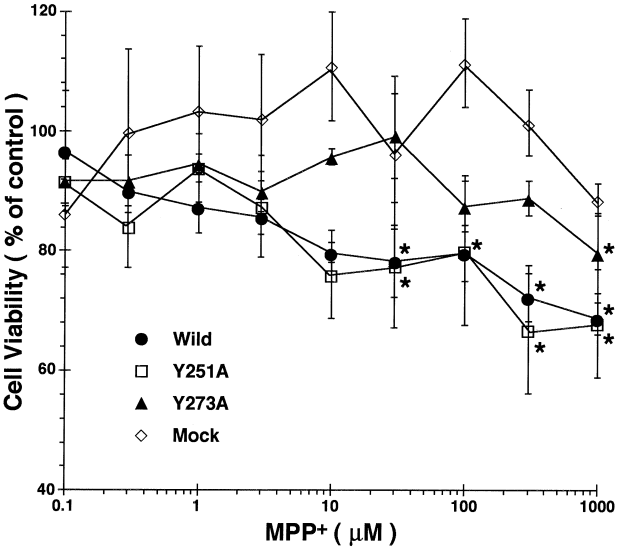

Fig. 5. Comparison of the MPP^+ toxicity in COS cells expressing the Y251A and Y273A mutant DAT to the wild-type. MPP^+ toxicity was assessed by counting cell number after incubation with MPP^+ using WST-1, and expressed as % of control (without MPP^+) in each cells expressing wild or mutant DATs. Points and bars represent the mean ± S.E.M., $n=4$. $^*P<0.05$ vs. control (without MPP^+).

Table 2
Relationship of reverse transport and MPP^+ toxicity in COS cells expressing wild or mutant rat dopamine transporters

Transporter	[^{3}H]MPP^+ uptake[a]		[^{3}H]MPP^+ efflux[b]		MPP^+ toxicity[c]
	K_m (μM)	V_{max} (fmol/μg protein/min)	(% of initial content/2 min)	(ΔWT)	
Experiment 1					
Wild	16.36 ± 2.70	1.00	18.69 ± 0.68		
7S-A2	14.80 ± 2.19	3.18 ± 0.53	24.22 ± 0.733	+5.53	↓
Experiment 2					
Wild	38.0 ± 5.1	1.00	30.92 ± 1.48		
Y251A	9.0 ± 1.1	0.16 ± 0.01	27.53 ± 0.49	−3.39	→
Y273A	35.2 ± 4.2	0.27 ± 0.01	37.47 ± 2.07	+6.55	↓
Experiment 3					
Wild	19.74 ± 4.34	1.00	31.28 ± 1.12		
Y533A	9.48 ± 1.96	0.81 ± 0.10	32.225 ± 2.04	+0.94	→
Y533F	50.94 ± 11.57	2.26 ± 0.51	39.29 ± 0.98	+8.00	↓

[a]Expt. 1 was from [13] and Expt. 3 was from [15].
[b][^{3}H]MPP^+ efflux was determined during initial 2-min incubation after loading and washing the cells, and expressed as % of loaded [^{3}H]MPP^+. Values are the mean ± S.E.M, $n=3$–4.
[c]MPP^+ toxicity was expressed by comparing with COS cells expressing wild DAT (WT). ↓, decreased sensitivity below WT; →, similar sensitivity to WT.

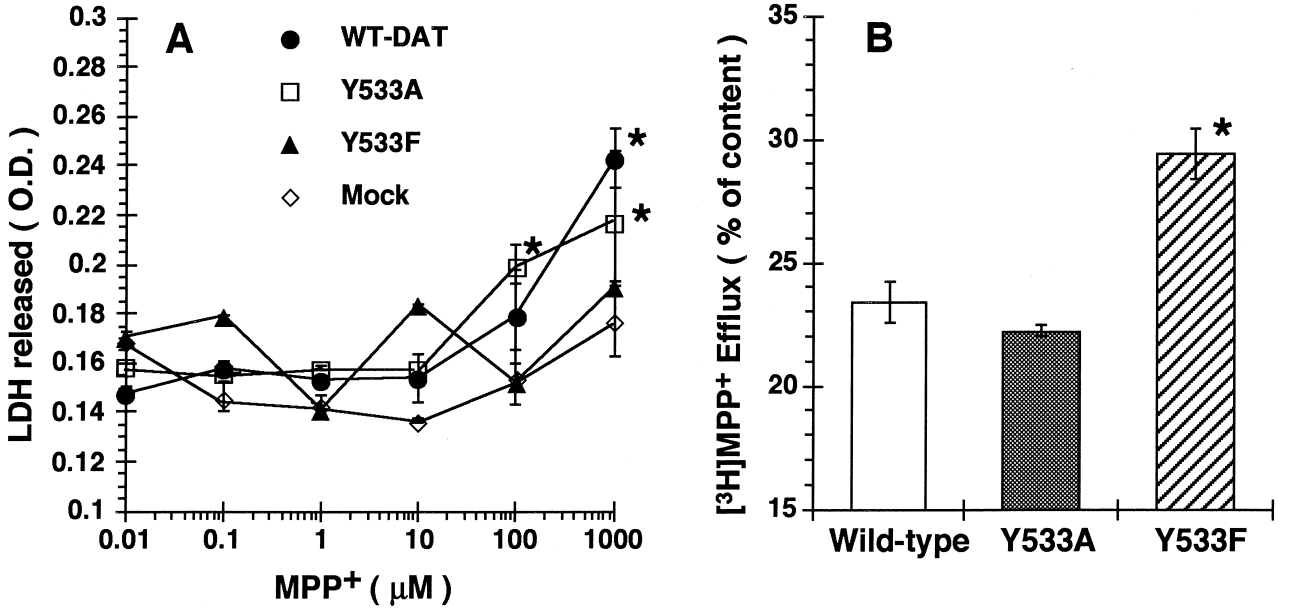

Fig. 6. MPP^+ toxicity (A) and [^{3}H]MPP^+ efflux (B) in COS cells expressing wild or Y533 mutant DATs. (A) After 1 h incubation of cells with various concentrations of MPP^+, cells were washed and incubated with fresh medium without MPP^+ for 3 h. LDH release into the medium during the latter stage of incubation was determined. Points and bars represent the mean ± S.E.M., $n=4$. $^*P<0.05$ vs. control (without MPP^+). (B) COS cells expressing wild or mutant DATs were preloaded with [^{3}H]MPP^+. After washing, cells were incubated with fresh medium for 2 min, and the efflux of radioactivity was determined by liquid scintillation counting. Values represent the mean ± S.E.M., $n=3$. $^*P<0.05$ vs. wild-type (WT).

pressing Y251A and Y273A mutant DATs, but not significantly due to large variations. However, the B_{max} values for [^{3}H]CFT binding did not change, suggesting that level of expression in mutant DATs was normal. Sensitivity to MPP^+ toxicity in COS cells expressing Y251A mutant DAT was similar to that in COS cells expressing wild DAT, while toxicity in COS cells expressing Y273A mutant DAT decreased (Fig. 5). Again, we examined the reverse transport of [^{3}H]MPP^+ in COS cells expressing these mutant DATs. [^{3}H]MPP^+ efflux through Y251A mutant DAT was similar to that through wild DAT, while that through Y273A mutant DAT slightly increased (Table 2).

Recently, we have demonstrated that the replacement of tyrosine-533 lying in the 11th putative transmembrane region by alanine (Y533A) causes an increased affinity for MPP^+ without changing V_{max}, whereas replacement by phenylalanine (Y533F) caused a decreased affinity with an increased V_{max} for MPP^+ [15]. COS cells expressing Y533A mutant DAT revealed a sensitivity to MPP^+ toxicity similar to that of COS cells expressing wild DAT (Fig. 6A). On the other hand, COS cells expressing Y533F mutant DAT revealed less sensitivity to MPP^+ toxicity. Furthermore, Y533F, but not Y533A, mutant DAT showed increased efflux activity (Fig. 6B).

Table 2 summarized the uptake and reverse transport of MPP^+ and the sensitivity to MPP^+ toxicity in COS cells expressing wild or mutant DATs. Even at the increased MPP^+ uptake velocity, if efflux was facilitated, MPP^+ toxicity decreased. The finding that even with the decreased V_{max} for MPP^+ uptake, increased affinity for MPP^+ uptake facilitates its toxicity suggest that affinity rather than velocity may be the critical factor in MPP^+ toxicity. Given all the above data, it is possible that re-distribution of MPP^+ due to influx/efflux turnover through the transporter may be important for MPP^+ toxicity expression.

4. Discussion

Systemic administration of MPTP causes selective cell death of dopaminergic neurons in the brain. There is a line of evidence indicating different sensitivities to MPTP between species. Species differences

in MAO_B activity, which catalyzes MPTP to a toxic metabolite, MPP^+, has been proposed as a mechanism affecting sensitivity to MPTP. Recently, Giovanni et al. [17,18] demonstrated that, despite similar levels of MAO_B activity in rat and mouse brain, mouse has greater sensitivity to MPTP toxicity than rat. They explained that although transport of MPP^+ to the dopaminergic neuron was approximately the same, longer term retainment of MPP^+ was observed in mouse brain, possibly due to slower clearance. This may be achieved by glial re-uptake of MPP^+, slower transport into vessels through the specific transporter on endothelial cells, or by some unknown mechanism. Transporter in plasma membranes of dopaminergic neuronal cells, i.e. DAT, may affect the clearance of MPP^+ from cytosol. We have demonstrated the bi-directional transport of DA and MPP^+ through DAT in COS cells and *Xenopus laevis* oocytes expressing rat DAT [11,12], suggesting that the reverse transport of MPP^+ via DAT might affect its cytosolic clearance and, thus, its toxicity.

In the present study, we examined the relationship between MPP^+ toxicity and DAT activity in COS cells expressing wild DAT or various mutant DATs, which showed different transport properties. Given the data presented here, we concluded that enhanced reverse transport of the toxin by DAT reduces sensitivity, suggesting that bi-directional transport via DAT may be an important factor for MPP^+ toxicity expression.

Pifl et al. [10] compared transport properties of human DAT and NET expressed in different cell lines to explain different sensitivities to MPTP among dopaminergic and adrenergic neurons. Despite different transport activity for different substrates, such as DA, norepinephrine and MPP^+, DAT showed similar uptake velocity for DA and MPP^+, while NET showed a lower MPP^+ uptake velocity than norepinephrine in contrast to the higher apparent affinity of NET for MPP^+ than DAT. They suggested from these results that the uptake velocity of MPP^+ is more important for its toxicity than its affinity. Contrary to this finding, we demonstrated that COS cells expressing mutant DAT which revealed a marked increase in uptake velocity, such as 7S-A2 and Y533F, shows less sensitivity to MPP^+ toxicity. Comparing affinity and uptake velocity for MPP^+ with toxicity revealed that increased velocity did not correlate well with toxicity, while decreased affinity (K_m) paralleled decreased sensitivity to MPP^+ toxicity. These results suggest that the affinity of the transporter may be a critical factor in MPP^+ toxicity. The reason for the discrepancy between Pifl's results and ours is unclear. Since they did not examine MPP^+ toxicity in the cells expressing the DAT and NET [10], we speculate that cell lines for expression of the transporter may affect MPP^+ toxicity, resulting from different levels of transport activity due to cell-specific environment for the transporter in the plasma membrane.

Our previous investigations [13–15] of the structure–function relationship of DAT by site-directed mutagenesis suggest that the MPP^+ recognition site might be different from the DA recognition sites, suggesting a possible strategy for developing a specific medicament for dopaminergic neuron toxins by preventing their transport without affecting DA uptake. In addition to the neuroprotective mechanism of vesicular monoamine transporter which has been demonstrated to sequester MPP^+ [19], the present study suggests that facilitation of toxin extrusion *via* DAT may attenuate its toxicity.

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